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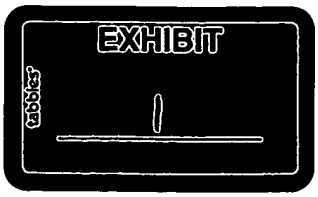

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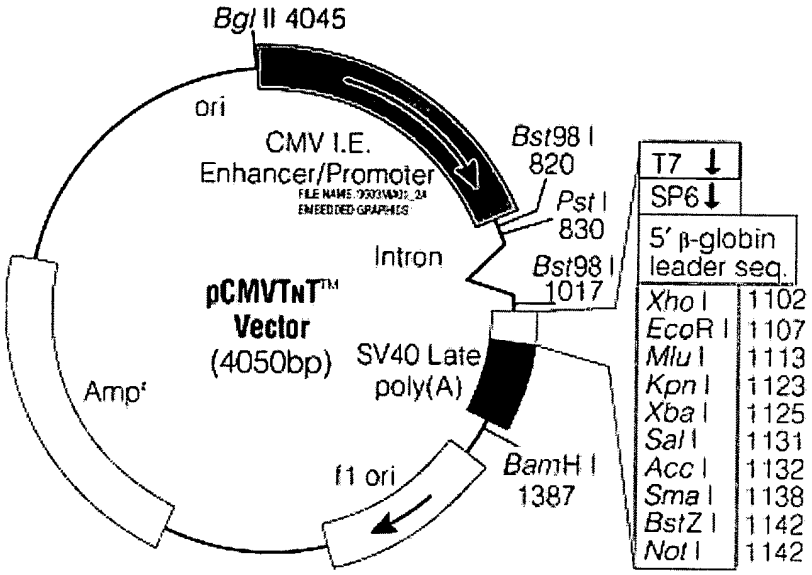

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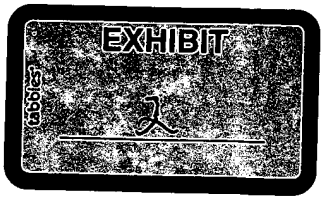

RESEARCH ARTICLE

CMV enhancer/human PDGF-β promoter for neuron-specific transgene expression

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Using cell-type-specific promoters to restrict expression of therapeutic genes to particular cells is an attractive approach for gene therapy, but often hindered by inefficient transcriptional activities of the promoters. Knowing the enhancer for the human cytomegalovirus (CMV) immediate-early gene improves activities of several cell-type- or tissue-type-specific promoters, we set out to investigate whether it improves neuronal transgene expression driven by a neuron-specific promoter, the platelet-derived growth factor B-chain (PDGF-β) promoter. A hybrid promoter was constructed by appending a 380-bp fragment of the CMV enhancer 5′ to the PDGF-β promoter. The plasmid containing the promoter was complexed with polyethylenimine for in vitro *and* in vivo *gene transfer. In cultured cells, the plasmid with the hybrid promoter significantly augmented expression of a luciferase reporter gene, providing expression levels 8- to 90-fold and 7- to 178-fold higher than those from two baseline constructs containing the PDGF-β promoter alone and the CMV enhancer alone, respectively. In particular, the activities of the hybrid promoter in two neural cell lines were close to or higher than that of the CMV immediate-early gene enhancer/promoter, a transcriptional control element that has been considered to be the most robust one identified thus far. After stereotaxic injection into the hippocampus and striatum in rats, the hybrid promoter displayed a neuronal specificity, driving gene expression almost exclusively in neurons. Transgene expression in the brain driven by the hybrid promoter was detectable 24 h after injection, being 10-fold higher than that driven by the PDGF-β promoter alone. The expression peaked around 5 days at 1.5 × 10⁵ relative light units per brain and lasted for at least 4 weeks. This differed strikingly from the expression driven by the PDGF-β promoter, which was no longer detectable on day 3. The new gene regulatory construct reported in this study will be useful to improve neuronal transgene expression required for gene therapy of neurological disorders and functional studies of the nervous system.*

Gene Therapy (2004) **11**, 52–60. doi:10.1038/sj.gt.3302126

Keywords: *CMV enhancer; PDGF-β promoter; neuron; plasmid; gene expression*

Introduction

Gene therapy has been considered as a potential approach to treatment of neurological disorders. To realize this goal, several obstacles to successful gene transfer that are related to unique attributes of the central nervous system (CNS) must be overcome. One of the obstacles is the great diversity of cell types in the CNS, many of which have critical physiological functions and are highly sensitive to change. This underscores the importance of restricting expression of a therapeutic gene to a particular type of cells in CNS gene therapy, thus ensuring therapeutic effects in the desired cells while limiting side effects caused by gene expression in nontarget cells. In view of the fact that the efficient targeted transduction of genes still represents a major barrier, the approach of restricting gene expression to a specific cell population through the use of a cellular promoter is particularly attractive.[1–3] In addition to offering cell-type-specific gene expression, the promoters, because of their cellular authentic sequences, may be less likely to activate host cell defense machinery, thus are usually less sensitive to cytokine-induced promoter inactivation than viral promoters. As such, the improved stability of gene expression can be expected.

The promoter for platelet-derived growth factor (PDGF) B-chain (PDGF-β promoter) is one such cellular promoter that may offer specific gene expression in neurons. PDGF B-chain is heavily expressed in neurons throughout the brain and the spinal cord, but not in glial cells.[4] Positive immunostaining of the polypeptide was observed in cytoplasmic, perinuclear regions and principal or secondary dendrites. The PDGF-β promoter has been used to direct the expression of transgenes to differentiated neurons in the cortex, cerebellum, brainstem, spinal cord and olfactory bulb in transgenic animals,[4] including mouse models for Alzheimer's disease overexpressing a mutated beta-amyloid precursor protein[5] and apolipoprotein E.[6] The promoter has also been used to facilitate neuron-specific transgene expression in virus gene delivery systems.[7,8]

One limitation to the applicability of cellular promoters, including the PDGF-β promoter, in gene therapy has been their relatively weak transcriptional activity compared with viral promoters, such as the enhancer and promoter for the human cytomegalovirus (CMV)

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Received 18 December 2002; accepted 16 June 2003

immediate-early gene.[9] The use of a cellular promoter together with a viral transcriptional regulatory element in gene transfer vectors provides a promising strategy to enhance expression of transgenes.[10–12] Knowing the CMV enhancer/promoter is the most robust transcriptional control element identified thus far, several previous studies had added a CMV enhancer region 5′ to a cellular promoter to increase its transcriptional activity.[13–17] However, expression from this kind of hybrid promoters could be influenced by the choice of cell-specific promoters and targeted tissue, and the feasibility of the approach in improving a neuronal-specific promoter in the nervous system has not been assessed. We set out to combine the advantages of the PDGF-β promoter and the CMV enhancer together in a hybrid promoter construct by appending a 380-bp fragment of CMV enhancer upstream of the 1.5-kb PDGF-β promoter. Our study demonstrates the preserved neuron specificity of the PDGF-β promoter after the CMV enhancer sequence was inserted, accompanied by an improved transgene expression for a relatively long period of time.

Results

Improved neuronal expression in cultured cells

We first cloned all gene regulatory elements into the same luciferase reporter vector, pGL3-basic vector (Promega, WI, USA), in order to eliminate the possible variations in gene expression levels caused by different plasmid backbones. These regulatory elements were obtained from pRc/CMV_2 (Invitrogen, CA, USA) and psubPDGF-EGFP, kindly provided by Professor H Büeler at the Institute of Molecular Biology, University of Zurich, Switzerland.[11] Four luciferase reporter plasmids were constructed, namely pCMV E/P with the CMV immediate-early gene enhancer/promoter, pCMV E with the CMV immediate-early gene enhancer, pPDGF with the PDGF-β promoter and pCMV E-PDGF with the CMV immediate-early gene enhancer/PDGF-β chain hybrid promoter (Figure 1a). Luciferase expression from pCMV E-PDGF was compared *in vitro* with those from the other three constructs following the transfection of neuronal (PC12 and C17.2) and non-neuronal (COS-7, KB3-1 and U251) cells with plasmid DNA complexed with polycation polyethylenimine (PEI).

As shown in Figure 1b, pCMV E-PDGF significantly increased gene expression when compared with pPDGF, by four-fold in U251, eight-fold in PC12, 13-fold in COS-7, 24-fold in KB3-1 and 90-fold in C17.2. When compared with pCMV E, the pCMV E-PDGF increased the expression seven-, 42-, 57- and 178-fold in KB3-1, C17.2, PC12 and COS-7 cells, respectively. The enhancement greatly exceeded the sum of the individual expression levels driven by the PDGF-β promoter and the CMV enhancer, indicating a synergistic effect of the two gene regulatory elements. Notably, in neuronal cells, expression levels derived from the hybrid promoter were close to or even higher than those of the CMV immediate-early enhancer/promoter, widely regarded to be the strongest known transcriptional control element. In non-neuronal COS-7 and KB3-1 cells and in glioblastoma U251 cells, the levels of expression from pCMV E-PDGF were 28, 21 and 2% of those from pCMV E/P, respectively. In neuronally differentiated PC12 and

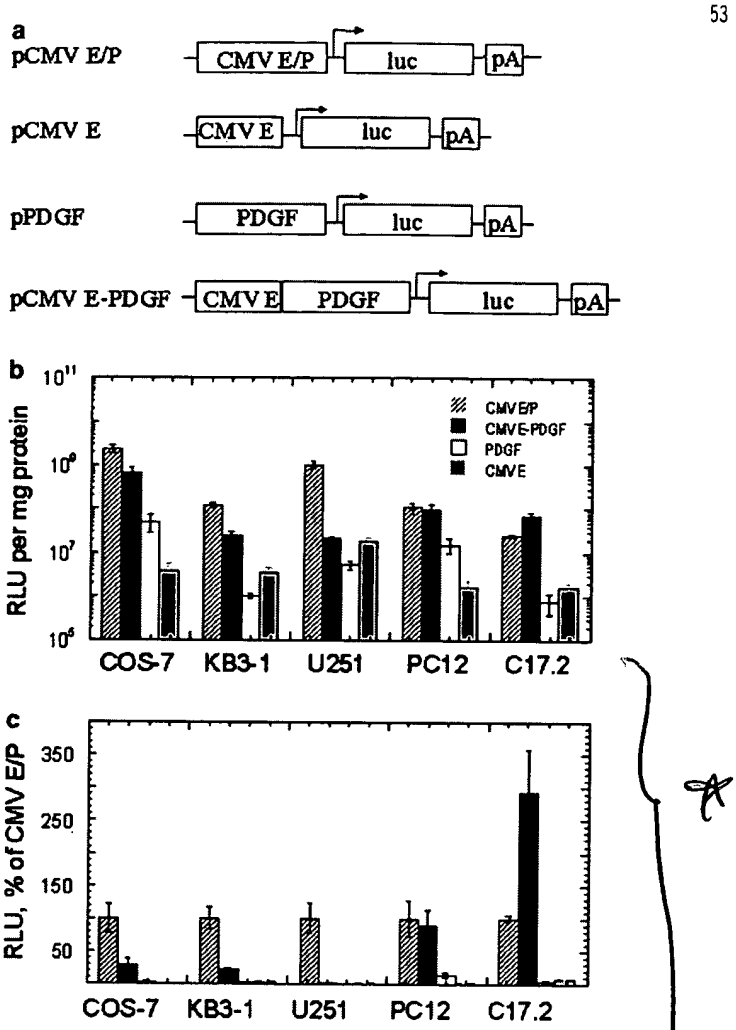

Figure 1 Activities of four different regulatory elements in cultured cells. (a) Schematics of the eucaryotic expression cassettes of the plasmids used in the study. Abbreviations: CMV E/P, CMV immediate-early gene enhancer/promoter; CMV E, the CMV immediate-early gene enhancer; PDGF, the PDGF-β promoter; CMV E-PDGF, the CMV immediate-early gene enhancer/PDGF-β chain hybrid promoter; luc, luciferase gene; pA, polyA tail. The above plasmids were used for transfection of neuronal (PC12 and C17.2) and non-neuronal cells (COS-7, KB3-1 and U251). The results are expressed as RLU/mg protein ± s.d. in (b) and percent of the readings from pCMV E/P in (c).

C17.2 cells, the corresponding percentages were much higher, at 88 and 292%, respectively (Figure 1c). The higher efficiency in cultured neuronal cells compared to non-neuronal cells indicates that the hybrid promoter has retained the neuronal characteristics of the PDGF-β promoter. Particularly, the difference between the neuronal cells and U251 suggests that the hybrid construct preferentially functioned in neurons over glial cells.

The retained neuronal characteristics of the hybrid promoter were further shown in a 7-day time-course study in neuronally differentiated C17.2 cells. When pCMV E/P or pCMV E was used for cell transfection, a rapid drop of expression levels by 50% within 3 days was observed (Figure 2). The profile of gene expression from pCMV E-PDGF was similar to that from pPDGF, retaining the initial expression level for at least 3 days (Figure 2b). A significantly high level of transgene

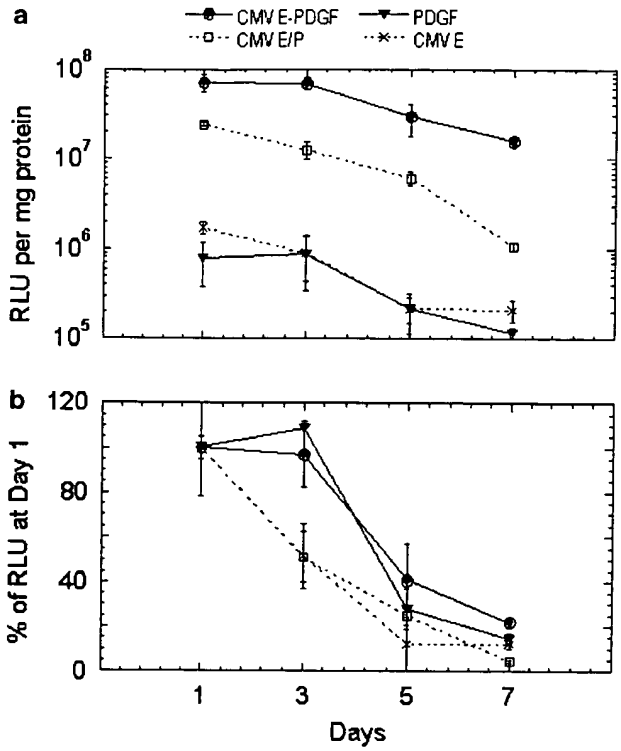

Figure 2 Time-course study in neuronally differentiated C17.2 cells. C17.2 cells were differentiated into neurons in a serum-free medium and transfected as stated in 'Materials and methods'. Four samples were used for each construct per time point. The results are expressed as RLU/mg protein ± s.d. in (a) and percent of the readings at day 1 in (b).

expression, around 1×10^7 RLU (relative light units) per mg protein, was still detectable on day 7 in the cells transfected with pCMV E-PDGF (Figure 2a). Owing to the limited survival time of the neuronally differentiated cells, no further examination was carried out beyond day 7.

Three reporter plasmids, pCMV E/P–EGFP, pPDGF-EGFP and pCMV E-PDGF-EGFP, were also constructed to drive the expression of the enhanced green fluorescence protein (EGFP). Using pPDGF-EGFP to transfect C17.2 neuronal cells, only a few EGFP-positive cells could be observed under a fluorescence microscope. The number of the positive cells was slightly larger when using pCMV E/P–EGFP. The highest number of EGFP-positive cells was obtained with pCMV E-PDGF-EGFP, accounting for about 20–40% of the total number of cells. Many neurons showed high levels of EGFP expression extending to the growth cones of their processes.

Neuron-specific expression of pCMV E-PDGF

We then investigated whether the CMV enhancer/PDGF hybrid promoter could maintain the neuronal specificity of the PDGF-β promoter in the rat brain. pCMV E-PDGF was complexed with PEI and injected into the rat hippocampus and striatum. For comparison, pCMV E/P was also used in this *in vivo* study. Quantitative analysis was carried out after double staining of brain tissue sections with antibodies against luciferase to visualize the transfected cells and antibodies against the neuron-specific nuclear protein (NeuN) to visualize neurons. In the hippocampus, cells expressing luciferase from pCMV E-PDGF were mostly in the pyramidal cell layer in the CA1 region and the granular cell layer in the dentate gyrus. Immunohistochemical colocalization of these cells with the NeuN marker confirmed that most of them were neuronal (Figure 3). Cell counting demonstrated that 89% of the cells in the hippocampus that expressed luciferase also contained NeuN (Figure 5). The pattern of expression from pCMV E/P differed significantly, with a diffuse anatomical distribution spread out from the neuronal cell layers (Figure 3). Many of the luciferase-expressing cells were positively stained using antibodies against the glial fibrillary acidic protein (GFAP), a specific marker for glial cells (data not shown). Quantitative analysis of cells expressing luciferase from pCMV E/P in the hippocampus showed that only 50% of the luciferase-stained cells were neurons (Figure 5). In the striatum (Figure 4), the cells expressing luciferase from pCMV E-PDGF were again restricted to neurons, while the pCMV E/P-transfected cells had a diffuse distribution, with many being GFAP positive (Figure 6). Cell counting revealed that 91% of luciferase-expressing cells from pCMV E-PDGF were neurons, significantly higher than the 48% of colocalization with pCMV E/P (Figure 5). However, the total number of luciferase-positive cells from pCMV E/P was significantly higher, about twice that from pCMV E-PDGF, in both the hippocampus and the striatum (Figure 5).

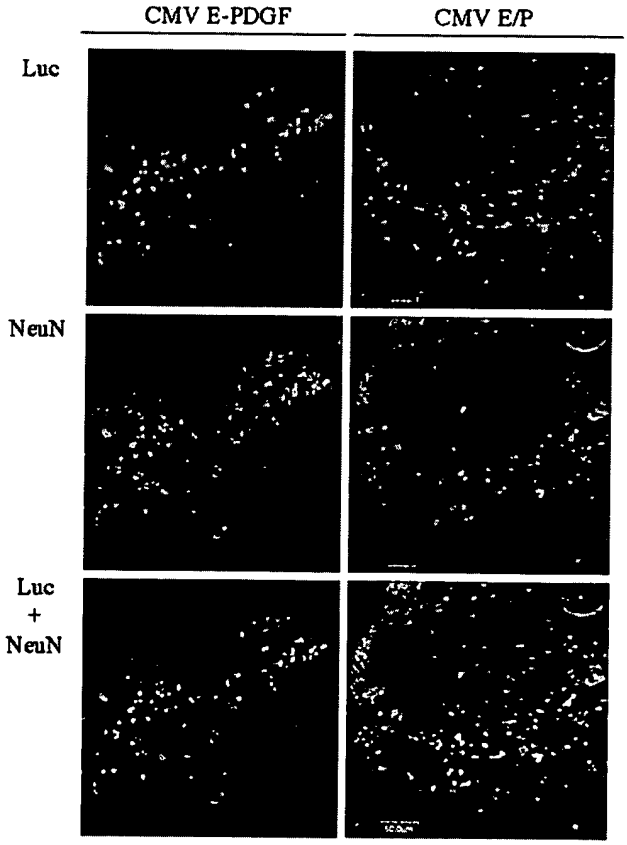

Figure 3 Confocal images of luciferase expression in neurons in the rat hippocampus. At 5 days after injection, rat brains were collected. Frozen coronal sections of each brain were cut at 30 μm thickness and used for double immunostaining against luciferase protein to show transfected cells and against neuron-specific nuclear protein (NeuN) to show neurons.

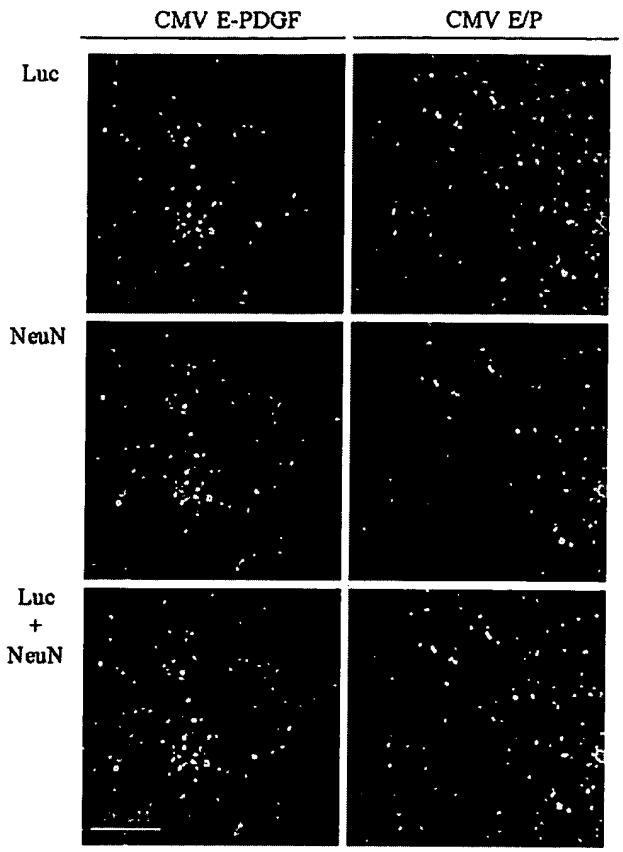

Figure 4 Confocal images of luciferase expression in neurons in the rat striatum. At 5 days after injection, rat brains were collected. Frozen coronal sections of each brain were cut at 30 μm thickness and used for double immunostaining against luciferase proteins to show transfected cells and against (NeuN) to show neurons.

Prolonged in vivo expression from pCMV E-PDGF

An *in vivo* time-course study was then carried out using pCMV E-PDGF and its control vectors, pPDGF, pCMV E and pCMV E/P, to analyze the expression kinetics of the plasmids. The plasmid constructs were complexed with PEI and injected into the striatum, and luciferase activities in the brain homogenates were quantified using a luminometer. Luciferase expression was detected in all the constructs as early as 24 h postinjection. However, expression levels from two control vectors, pPDGF and pCMV E, were significantly lower, being about 1/10 of that from pCMV E-PDGF in the case of pPDGF, and became undetectable by day 3 (Figure 7). The expression from pCMV E/P peaked at day 3 at 1.2×10^6 RLU per brain, then dropped to less than 1×10^4 RLU per brain by day 28 (Figure 7). The expression from pCMV E-PDGF was more stable, lasting for at least 2 weeks at a level around 1×10^5 RLU per brain. After 4 weeks, the reading decreased to 1/4 of the peak level, but was significantly higher than that produced by pCMV E/P (Figure 7). The expression became almost undectectable after 2 months.

Discussion

The flexibility in modifying DNA sequences in plasmids provides a straightforward and efficient way to investigate activities of gene regulatory elements. Our initial studies examined the efficiency of the PDGF-β promoter in the pGL3-basic vector to drive luciferase reporter gene expression in cultured neuronal or non-neuronal cells. The promoter showed a much lower transcriptional activity than viral promoters, such as the CMV enhancer/promoter, the RSV promoter and the SV40 promoter, and other cell-specific promoters, including elongation factor 1 α promoter and Tα 1 tubulin promoter (unpublished observations). By appending a CMV enhancer sequence 5′ to the PDGF-β promoter, the activity was enhanced significantly in both cultured neuronal cells and the rat brain, as shown in this study. Furthermore, the neuronal specificity of the PDGF-β promoter is preserved in the hybrid construct when used in the brain. Consistent with this, the activity of the

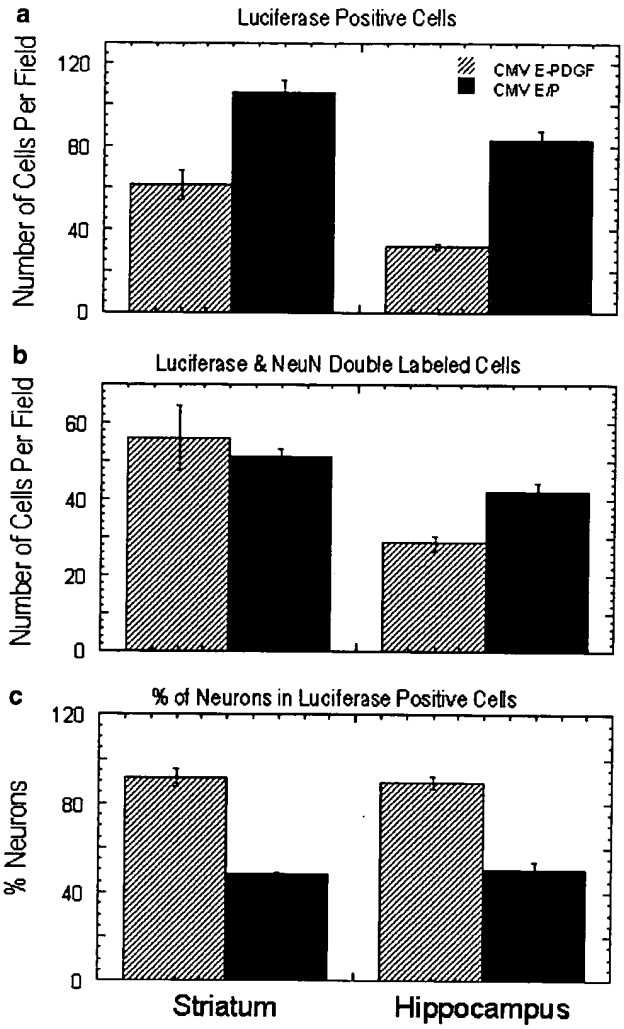

Figure 5 Neuronal specificity of the CMV E-PDGF hybrid promoter and the CMV enhancer/promoter. Quantitative analysis was carried out in tissue sections double stained with antibodies against luciferase protein and (NeuN). Three rats were used for each promoter. For each region, four sections per rat were selected randomly and three fields from each section were used for cell counting. The numbers of luciferase immunoreactivity positive cells that contain or lack the neuronal marker NeuN immunoreactivity were counted. (a) Luciferase-positive cells; (b) luciferase and NeuN double-labeled cells; and (c) percentage of transfected neurons=number of double-labeled cells/number of luciferase-positive cells.

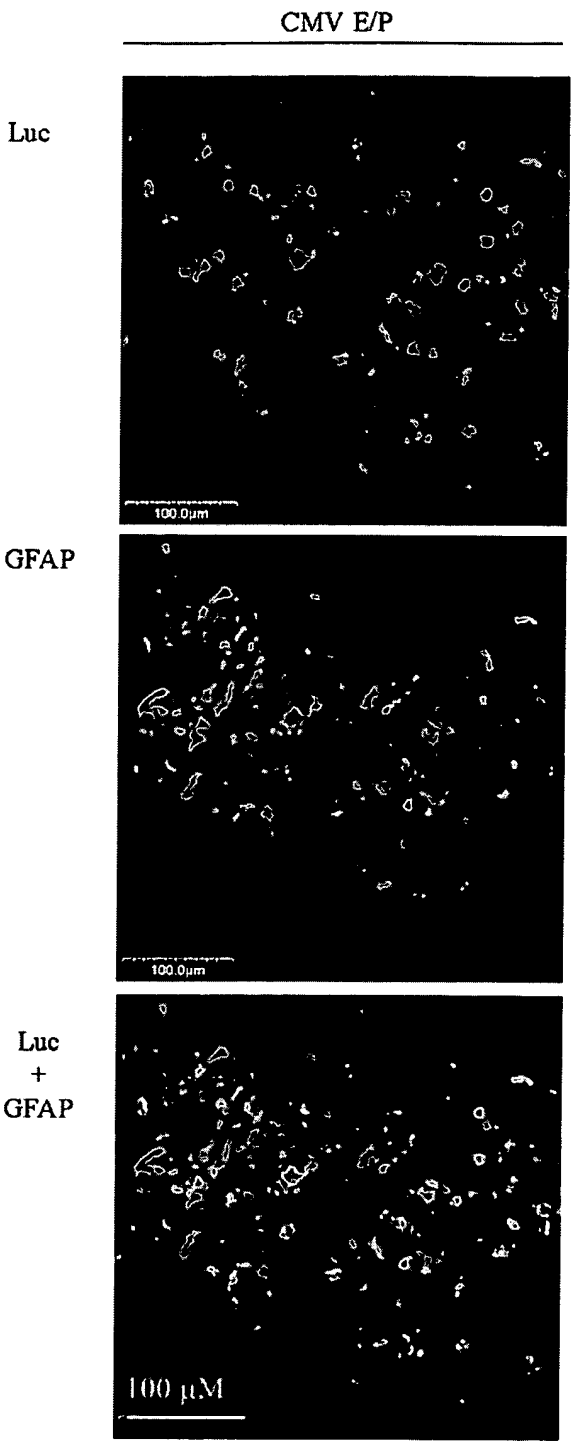

Figure 6 Confocal images of luciferase expression in GFAP-positive cells in the rat striatum. At 5 days after injection, rat brains were collected. Frozen coronal sections of each brain were cut at 30 μm thickness and used for double immunostaining against luciferase protein to show transfected cells, and against GFAP to show glial cells.

hybrid construct was detected only in ganglion neurons in the retina after vitreous body injection (unpublished observation). To our knowledge, this study is the first to use the CMV enhancer together with a neuron-specific promoter. The PDGF-β promoter is able to transduce

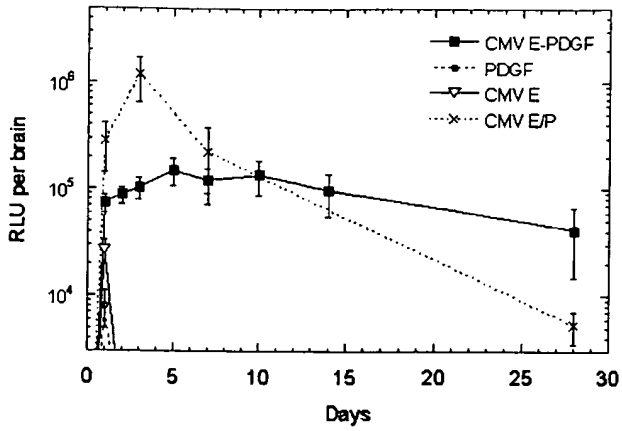

Figure 7 Time course of luciferase expression after stereotaxic injection of pCMV E-PDGF, pCMV E, pPDGF or pCMV E/P into rat striatum. DNA was complexed with PEI and injected into the striatum at three injection points as stated in 'Materials and methods'. For each injection, 5 μl of the complexs containing 1 μg DNA was used. Brain samples were collected at various time points and homogenized by sonication. Supernatants at room temperature were used for luciferase assay. Four rats were used per time point. The values are presented as means ± s.d.

significantly more dopaminergic neurons, when used in a recombinant adenoassociated virus vector, than titer-matched vectors carrying the CMV enhancer/promoter.[11] The CMV–PDGF hybrid promoter developed in this study may further improve gene transfer in these neurons, offering a potential tool for the treatment of Parkinson's disease and other neurological disorders.

Our *in vitro* study showed gene expression levels derived from the CMV enhancer–PDGF hybrid promoter close to or even higher than that of the CMV enhancer/promoter in neuronal cells. However, the CMV enhancer/promoter in the brain still displayed an activity, at its peak level, at least two-fold higher than the hybrid promoter. The difference between *in vitro* and *in vivo* findings was most likely caused by the activity of CMV enhancer/promoter in non-neuronal cells. As shown in our *in vitro* experiments and *in vivo* quantitative analysis, pCMV E-PDGF activity was lower in glial cells. Assuming that PEI/DNA complexes have the equal opportunity to enter neurons and non-neuronal cells in the brain, in the case of using pCMV E-PDGF, only neurons will express the transgene, while under circumstances of using pCMV E/P, both neurons and non-neuronal cells will show gene expression. It has been reported that combined transductional and transcriptional targeting improves the specificity of transgene expression.[18] We would expect a further improved gene expression from our plasmid containing the hybrid promoter when used in a targeted gene delivery system, which would enhance the entry of the plasmid into desired cells, for example neurons, and reduce the uptake of the plasmid by nontarget cells.

One finding from the current study was a sustained gene expression in neurons for at least 4 weeks driven by the CMV enhancer–PDGF hybrid promoter. The CMV enhancer or PDGF-β promoter alone did not confer sustained expression with the nonviral delivery system used. In previous studies, the CMV enhancer/promoter activity varied with the use of different delivery systems. When used in nonviral systems and some of viral

systems, it allowed for a very strong short-term expression of transduced gene and became silent within a few weeks after gene transfer.[19–22] When used in PEI/DNA complexes for gene transfection in the brain, the CMV enhancer/promoter drove an initial higher level of gene expression that was maintained for 1 week or so and then rapidly declined by orders of magnitude,[23] which was observed as well in the current study. Promoter inactivation rather than loss of vector DNA has been suggested to be the primary factor limiting long-term gene expression.[17,20] Possible causes include hypermethylation of promoter and depletion of endogenous activating transcription factors.[24,25] Cellular promoters are usually less sensitive to inactivation than viral promoters, as already observed with adenoviral and retroviral vectors.[9,26] The hybrid CMV–PDGF promoter reported here conferred relatively longer-term expression of the transgene, suggesting that the set of transcriptional factors interacting with the hybrid promoter may override the normal inactivation of the PDGF-β promoter and thus maintain activity. Long-term expression by combining the CMV enhancer or enhancer/promoter with other regulatory elements, mainly muscle-specific promoters, has also been observed by other investigators.[17,27,28]

The mechanism for the increased transcriptional activity derived from the CMV–PDGF hybrid promoter is as yet unknown. It is well recognized that enhancer and promoter recognition by RNA polymerase, transcription factors and auxiliary proteins is a complex process, involving both primary and secondary sequence characteristics of the regulatory DNA. The number, diversity, orientation and placement of transcription factor-binding sites within promoters are critical parameters that define gene expression. Functional positive-regulatory transcription factor-binding sites in the PDGF-β promoter include those for Sp1, Sp3, Egr-1, NF-κB and others.[29–32] The CMV enhancer sequence used in our CMV–PDGF hybrid promoter avoids some known repressor binding sites for the cellular Gfi repressor, but still contains sites for the repressor YY1. From the experiments described and the results reported in this study, we can only speculate that the combination and arrangement of factor-binding sites in the CMV–PDGF hybrid promoter favors the synergistic interaction between regulatory DNA, transcription factors, accessory proteins and RNA polymerases in neuronal cells.

The current study was performed using plasmid DNA vectors. The CMV–PDGF hybrid promoter may also be useful in the context of viral vectors. The relatively small size of the hybrid promoter, 1.8 kb, implies that it is feasible to use it in adenoassociated virus vectors. However, plasmid DNA vectors are quickly gaining recognition as an alternative to viral vectors for their potentials in avoiding immunogenicity and toxicity problems inherent in viral systems.[33] The advantages of using plasmid vectors include lack of immunogenicity, especially when their CpG contents have been minimized, minimal safety concerns and considerable flexibility in terms of size and content.[34–36] The last feature is of particular interest in the use of cell-type-specific promoters, which could be too large to be used in many virus vectors. The gene transfer approach using plasmids has been greatly improved and efficient transgene expression has been obtained in many tissues and organs. The increased, sustained transgene expression obtained in neurons with the polymer/DNA complexes containing our CMV–PDGF hybrid promoter represents a step towards the development of effective plasmid-based gene delivery systems for nonviral gene therapy in the brain and the spinal cord. However, the activity of the plasmid vector with the CMV–PDGF promoter still needs to be further enhanced before it becomes suitable for use in clinical trials. Other regulatory sequence elements within plasmid vectors, such as introns, translation initiation sites and the polyadenylation signal can be optimized to achieve this goal.

In conclusion, our study presents an efficient strategy to improve the activity of a weak neuron-specific promoter, supporting the hypothesis that functional promoters composed of multiple DNA elements can be designed to overcome the problems of nonspecific or weak expression profiles.[11,37–39] As the understanding of promoter structure and function grows, it should become possible to identify short, 100–200 bp, functional promoter elements, and to design compact combinations of these elements to achieve efficient, sustained and specific expression of transgenes.[40]

Materials and methods

Plasmids

pGL3-basic vector was purchased from Promega (Madison, WI, USA). PsubPDGF-EGFP[11] was kindly provided by Professor H Büeler (Institute of Molecular Biology, University of Zurich, Switzerland). The luciferase constructions of pCMV E-PDGF-luc, pCMV E-luc, pPDGF-luc and pCMV E/P-luc were based on pGL3-basic vector. To generate pCMV E-PDGF, the PDGF-β promoter and CMV immediate-early enhancer (−568 ~ −187 relative to the TATA box) were amplified from psubPDGF-EGFP and pRc/CMV$_2$ using PCR and inserted between *Sac*I/*Hind*III and *Kpn*I/*Sac*I, respectively, in the multiple cloning sites of the pGL3-basic vector. pPDGF and pCMV E were constructed similarly by introducing only the PDGF-β promoter insert or the CMV immediate-early enhancer insert, respectively. To construct pCMV E/P-, the CMV full-length promoter (−655 ~ +55 relative to the TATA box) was amplified from pRc/CMV$_2$ and inserted into *Kpn*I/*Hind*III sites of the pGL3-basic vector. Schematic structures of the four different plasmid vectors used in this study are shown in Figure 1a. Oligonucleotides used for PCR amplification are listed as follows: CMV E: sense primer 5′-ATTCGGTACC CCTGGGTCG ACATTGA-3′, antisense primer 5′-CAACGAGCTC AC CATGGTAATAGCGATG-3′; CMV E/P: sense primer 5′-ATTAGGTACC CGATGTACGGGCCAGATATACG-3′, antisense primer 5′-TAATAAGCTT ACTAGTGGATCCGAG CTCGGTA-3′; PDGF-β promoter: sense primer 5′-AATT GAGCTCCTAGAGGATCCACAGTCT-3′, antisense primer 5′-CAGCAAGCTTTTCAGTTCCTCGACTCTAG-3′.

All plasmid DNA was amplified in DH5α *Escherichia coli* and purified according to a standard protocol (Qiagen, Hilden, Germany). Plasmids obtained by PCR amplification were verified by DNA sequencing.

Preparation of DNA/PEI complexes

Plasmid DNA was diluted in 5% glucose. PEI (25 kDa; Sigma-Aldrich, San Diego, CA, USA) was used as an

aqueous stock solution containing 10 mM nitrogen. Ratios of PEI to DNA used for cell transfection and animal experiments were 10 and 14 equivalents of PEI nitrogen per DNA phosphate, respectively. The required amount of PEI was calculated by taking into account that 1 μg of DNA contains 3 nmol of phosphate, and that 1 μl of 10 mM PEI holds 10 nmol of amine nitrogen. Complexes were formed by adding the appropriate amount of PEI solution into the DNA solution, briefly mixing by vortexing and waiting for 30 min at room temperature.

Cell cultures

The *in vitro* transfection experiment was performed in neuronal (PC12 and C17.2) and non-neuronal cells (COS-7, KB3-1 and U251). PC12 rat pheochromocytoma cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 5% horse serum. PC12 cells were differentiated at a density of 5×10^4 cells/well in 24-well plates coated with 0.2 mg/ml rat-tail collagen in serum-free RPMI-1640 medium containing 50 ng/ml nerve growth factor (NGF) for 10 days. The percentage of cells possessing neurites was about 90%. C17.2 is a neural stem cell line generated via retrovirus-mediated v-myc transfer into murine cerebellar progenitor cells.[41] C17.2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate and 10% FBS. The cells were differentiated by plating them onto poly-L lysine (50 mg/ml)- and laminin (20 mg/ml)-coated 48-well plates at a density of 2×10^4 cells/well in serum-free DMEM/F12 media with 1% N2 supplement (Invitrogen, Netherlands) for 2 days. Under these differentiation conditions, over 90% of the cells display neuronal morphology and are positively stained using antibodies against 68 kDa neurofilament protein. COS-7, KB3-1 and U251 cells were cultured in DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were maintained in a 37°C, 5% CO_2, humidified incubator.

Gene transfection in vitro

For the transfection of COS-7, KB3-1 and U251 cells, the cells were split 1 day prior to transfection and plated in 24-well plates at a cell density of 5×10^4 per well with 0.5 ml of the above indicated medium. After overnight incubation, the culture medium was replaced with 300 μl of Opti-MEM (Invitrogen, Netherlands), and an aliquot of 10 μl of the DNA/PEI complexes containing 0.5 μg DNA was added to each well. DNA/PEI complexes were incubated with the cells for 3 h at 37°C. The medium was replaced with 0.5 ml of fresh complete medium and cells were further incubated for 24 h. For the transfection of neuronally differentiated PC12 and C17.2 cells, the cells were maintained in respective serum-free media and transfected with the DNA/PEI complexes containing 0.5 μg DNA in a 24-well plate for PC12 cells and the complexes containing 0.25 μg DNA in a 48-well plate for C17.2 cells. After the incubation, cells were washed and permeabilized with 100 μl of reporter cell lysis buffer (Promega, WI, USA). The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega, WI, USA). The RLU were normalized by the total protein concentration of the cell extracts, measured using a protein assay kit (Bio-Rad, Hercules, CA, USA).

Animals

Adult male Wistar rats (weighing 250–320 g) were used in this study. For luciferase activity studies, four rats were used for each time interval. For immunohistochemical study, six rats were used per group for sham-operated group, pCMV E-PDGF-luc injected group and pCMV E/P luc injected group. They were kept four to a cage in a light–dark cycle (12 h/12 h) at a constant temperature of 22°C and at 60% humidity, and fed with normal laboratory rat food. In the handling and care of all animals, the International Guiding Principles for Animal Research as stipulated by World Heath Organization (1985) and as adopted by the Laboratory Animal Center, National University of Singapore were followed. All rats were bred and supplied by the Laboratory Animal Center, National University of Singapore, and kept in the animal unit before and after the experiments.

Gene transfection in vivo

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg of body weight) and positioned in a stereotaxic instrument with the nose bar set at 0 (for striatum injection) or −3.5 mm (for hippocampus injection). DNA/PEI complexes were separately, bilaterally injected into the striatum at three injection points. The coordinates from bregma and dura were: anterior (A), +1.5 mm, lateral (L), +2.0 mm, ventral (V), −5.0 mm; A, +0.5 mm, L, +3 mm, V, −5.0 mm; and A, −0.3 mm, L, +3 mm, V, −5.0 mm. DNA/PEI complexes were also injected into the hippocampus at the coordinates: A, −4.4 mm, L, +3.2 mm, V, −2.5 mm. A volume of 5 μl of the complexes containing 1 μg DNA was used in each injection. The rate of injection was 1 μl/min and the needle was allowed to remain *in situ* for 5 min before being slowly retracted at the end of each injection.

For immunohistochemical analyses, rats were killed 2 days after injection. Following deep anesthesia, all rats were perfused first with Ringer's solution followed by 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the cerebral cortices containing the striatum and hippocampus were removed and postfixed in the same fixative for 2–4 h before they were transferred into 0.1 M PBS containing 15% sucrose and kept overnight at 4°C. Frozen coronal sections of each brain were cut at 30 μm thickness and stored in 0.1 M PBS.

Free-floating sections were washed for 20 min in 0.1 M PBS at pH 7.4 containing 0.2% Triton X-100, then blocked with 5% normal goat serum in PBS for 1 h. Sections were then incubated overnight with polyclonal anti-luciferase (Promega, WI, USA; dilution 1:150) and a monoclonal antibody against NeuN (Chemicon International, USA; dilution 1:500). Sections were washed in 0.1 M PBS and further incubated with anti-rabbit IgG Tritc conjugate (Sigma-Aldrich, USA; dilution 1:100) and anti-mouse IgG Fitc conjugate (Sigma-Aldrich, USA; dilution 1:100) for 1 h. After incubation, sections were washed three times in PBS. They were then collected on gelatin-coated slides, mounted with DAKO fluorescent mounting medium, and covered with coverslips. Control sections were incubated without primary antibodies. Sections were

examined with a Carl Zeiss LSM410 confocal laser scanning microscope. Each section was initially scanned with a 488 nm laser line, and an emission filter BP 510–525 for the detection of Fitc fluorescein, then with a 543 nm laser line and an emission filter LP 570 for the detection of Tritc fluorescein.

For quantitative analysis of immunostained sections, three rats from each group were used to examine colocalization of pCMV E-PDGF-luc or pCMV-luc with NeuN. Four sections for each rat were selected randomly and used for cell counting. A total of three bilateral fields selected randomly were surveyed in each section. Each field was captured under a Carl Zeiss LSM410 confocal laser scanning microscope at a magnification of $\times 200$. Colocalized cells in each picture were counted, and percentages of colocalization of NeuN in pCMVIE-PDGF-luc or pCMV-luc-positive cells were calculated. Student's *t*-test was used to determine the statistical significance of the extent of colocalization with different plasmids.

For luciferase activity assays, rats were killed by intracardiac perfusion with 0.1 M PBS (pH 7.4) following deep anesthesia. The brain tissue samples were removed and stored at −80°C until processing. After adding PBS buffer (100 µl PBS per 50 mg tissue), each sample was homogenized by sonication for 10 s on ice, and then centrifuged at 13 000 r.p.m. at 4°C in a microcentrifuge. In total, 10 µl of the supernatant at room temperature was used for the luciferase activity assay employing an assay kit from Promega. The measurements were made in a single-well luminometer (Berthold Lumat LB 9501) for 10 s.

Acknowledgements

This work was funded by the Agency for Science, Technology and Research (A* STAR), Singapore. We thank Ms Shan Shan Wu for critical review of the manuscript, and other members in the lab for their helpful discussion and assistance.

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